Spring 5-12-2017

A COMPARATIVE STUDY OF MICROBIAL COMMUNITY OF UNDISTURBED AND RECLAIMED SOIL IN SOUTHEAST KANSAS

Ghaidaa Allhyani
Pittsburg State University, gallhyani@gus.pittstate.edu

A COMPARATIVE STUDY OF MICROBIAL COMMUNITY OF UNDISTURBED AND RECLAIMED SOIL IN SOUTHEAST KANSAS

A Thesis Submitted to the Graduate School in Partial Fulfillment of the Requirements for the Degree of Master in Science

Ghaidaa Allhyani

Pittsburg State University

Pittsburg, Kansas

July 2017

A COMPARATIVE STUDY OF MICROBIAL COMMUNITY OF UNDISTURBED AND RECLAIMED SOIL IN SOUTHEAST KANSAS

Ghaidaa Allhyani

APPROVED:

Thesis Advisor _____
Dr. Dixie Smith, Biology

Thesis Co-Advisor _____
Dr. Anuradha Ghosh, Biology

Committee Member_____
Dr. Philip Harries, Biology

Committee Member_____
Dr. Kevin Elliott, Technology

Acknowledgments

I would like to thank my advisor Dr. Dixie Lee Smith and my co-advisor Dr. Anuradha Ghosh for their unmeasurable support during the research and compilation of this thesis. They were always available when I needed their support and guidance. They provided me with guidelines towards developing my own original work by steering me to the right direction through prompt replies to my questions and request for guidance. Their utmost support directed my efforts towards producing a professionally crafted thesis.

I would also like to acknowledge the members of the thesis committee. Specifically, I would like to thank Dr. Philip Harries and Dr. Kevin Elliot for their high level of professionalism and skepticism that raised the bar higher for me to address issues clearly and concisely. Their passionate participation pushed me to produce at my level best. They showed their commitment through reading the thesis and providing me with valuable comments that facilitated my final work. Also, I appreciate the assistance provided by other graduate students, my brother Abdullah, Rachel and Abrar, in Dr. Ghosh's research laboratory.

I would also like to thank the Graduation and continuous studies at the Pittsburg state University for the opportunity to present my capability in taking this academic and professional leap. I would also like to thank the Saudi cultural mission whose work ensures that the country has a continuous flow of qualified and well-educated graduates. Through their support, I am part of that continuous flow of graduates.

Most importantly, I would like to thank my family members who have provided me with moral and physical support in my quest to complete this thesis. I would express my profound gratitude to them for their continuous encouragement throughout the process.

This accomplishment would not have become a reality without the help, guidance, and support of the aforementioned persons. Thank you.

A COMPARATIVE STUDY OF MICROBIAL COMMUNITY OF UNDISTURBED AND RECLAIMED SOIL IN SOUTHEAST KANSAS

An Abstract of the Thesis by
GHAIDAA ALLHYANI

The purpose of this project is to compare the microbial communities of the 33 year-old reclaimed soil at the Monahan with the original soil at the O'Malley. The data collected in this project investigates whether there is a correlation between soil age and the soil microbial community. Using soil DNA analysis is one way we can compare soil microbial communities. Soil DNA was isolated from multiple samples from two sites (reclaimed and an undisturbed soils) using DNA isolation kit. Using the community DNA as a template, eubacterial, archaeal, and fungal DNA fragments were PCR amplified using specific-primers. Attempts were made to clone the purified amplicon. Another approach based on culturable isolates was also followed. In parallel to the culture-independent molecular technique, the soil samples from the undisturbed site processed to identify bacterial isolates on culture media. The isolates obtained were compared to already isolated and identified bacterial isolates from the reclaimed site using restriction-fragment length polymorphism. The bacterial concentration was also compared with that of reclaimed soil samples. In addition, some isolates were tested for antibiotic resistance profile. The outcome of this project provides an initial attempt at characterizing the relationship between soil development and a microbial community. We found that there are no differences in the *bacterial* community of the 33-year-old reclaimed soil at the Monahan with the original soil at the O'Malley.

Table of Contents

List of Tables

List of Figures

Chapter I

Introduction

Microorganisms play important ecological roles which involve the maintenance of the ecosystem function and the nutrient cycling processes. Soil contains an estimated of 109 prokaryotes which is more than 2000 genome types per every gram of soil (Zhao et al, 2011)Today, laboratory-based techniques are making it possible to determine the molecular characterization of the communities by comparing the nucleic acids. Furthermore, the technique can create predictions about the natural evolutionary relationships by creating a classification scheme. These techniques include: 1-the tools that is in use to study microbial diversity is the Restriction Fragment Length Polymorphism (RFLP) which relies on DNA polymorphisms (Philippot et al, 2001). The methods help into the determination of intra-species variations together with structural changes in the microbial communities.2- Another method is Stable Isotope Probing which operates with nucleic acid methods (Philippot et al, 2001). One of the functions of this method is to link the identity of bacteria together with their essential function in the environment. 3-The Terminal-Restriction Fragment Length Polymorphism is another method that provides a clear picture of the community with diversity and phylogenetic details. Additionally, the method is also critical in the analysis of functional genes like those encoding for methane oxidation and nitrogen fixation (Philippot et al, 2001). 4- Fluorescence in Situ Hybridization (FISH) detects genes of microorganisms using DNA probes that are fluorescently labeled (Philippot et

al, 2001). Furthermore, FISH is also advantageous in detecting active cells by targeting rRNA thus providing information on the substrate used in complex microbial soils. 5- The Microarray method relies on the target molecules together with the combination of probe types. The method has three categories that help in the direct detection of rRNA (Philippot et al, 2001). 6- The Random Amplified Polymorphic DNA which requires very minimal sample materials together with obtaining the result quickly. Furthermore, the method uses DNA products by PCR which formulate their basis on random priming of the target DNA.

Finally, DNA Fingerprinting via gel electrophoresis helps distinguish the differences in genetic makeup of microbial from different samples. It is essential since it enables high sample throughput together with targeting sequences which are functionally significant (Zhao et al, 2011) (Scheidegger et al 2009).

One of the challenges to working with soil DNA is that of culturing majority of the soil organisms that are obtained from the environmental sample. It is difficult to culture soil microbes because the inability to culture became the biggest limitation to the understanding of soil ecology and microbial diversity (Wakelin et al, 2008). To overcome the limitation, special DNA-based techniques have been developed to extract soil microbes. These techniques allow environmental samples to be monitored directly so that the fate of particular genes or organisms can be monitored (Wakelin et al, 2008). One of the big problems with extracting soil DNA is that they were often contaminated with humic acids, these contaminants will interfere with the subsequent molecular biological manipulation. This technique was also very expensive and required a larger sample. Over the years, emerging tools have made for relatively easier and efficient techniques for DNA extraction from the soil microbes. New techniques involving minimal purification before the PCR amplification have helped to recover microbial

diversity, detection of rare species and discrimination of soil bacteria composition (Ahmad et al, 2011). In this project, PCR was utilized to amplify a piece of DNA. In every situation, PCR increase the number of copies DNA for further analysis (Yeates et al, 1998).

The efforts have shown that microbes are abundant and diverse in various types of soils. Samples obtained from reclaimed soils are expected to have different microbial compared with soil from un-reclaimed soils (Narendrula-Kotha & Nkongolo, 2017).

Restriction Fragment Length Polymorphism (RFLP) is a technique that exploits the homologous difference in DNA. The technique uses the presence of fragments of various sizes after digesting DNA samples with specific restriction endonucleases for the targeted sample. RFLP allows the individual soil to be identified relying on its unique pattern of enzyme restriction in a specific DNA region. This technique uses polymorphisms in the samples' genetic codes. Irrespective of the fact that members of the same species, symbolically same soils might have the same genetic makeup, the slight difference can account for the difference in phenotypes, such as the soil color and appearance thus enabling the use of RFLP for sampling (Scheidegger et al ,2009). The technique involves cutting a particular region on the targeted DNA (with known variability) with a restriction enzyme, after which the DNA fragments are separated by agarose gel electrophoresis. The entire RFLP process needs probe labeling, DNA fragmentation, electrophoresis, blotting, hybridization, washing in addition to autoradiography (Bluth & Bluth, 2013). The identified or detected RFLP can then be visualized using x-ray film in autoradiography where the DNA fragments can be viewed and analyzed accordingly.

Even though RFLP is effective in its use, the analysis process is tedious and slow. The process requires a large sample of DNA; additionally, the process from probe

labeling to washing and autoradiography takes more than 20 days to complete. RFLP markers are dominant and highly locus specific thus preferable for DNA sampling. The purpose of this project is to compare the microbial communities of the 33-year-old soil at the Monahan with the original soil at the O'Malley. The data collected in this project investigates whether there is a correlation between soil age and the soil microbial community. We expect that there would be a difference in the microbial community of a relatively undisturbed, original soil and the newer soil. The aboveground vegetation is less divers in the 33-year-old soil, and dominated by switchgrass. We expected the belowground biota to be similarly less diverse than in the original.

Chapter II

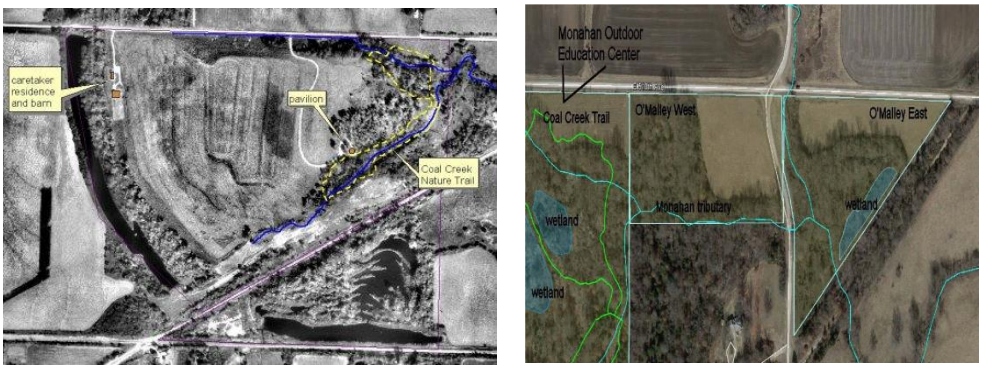
Materials and Method:

Sampling Procedures

Field Sampling:

Materials for this research were equipped by the PSU Department of Biology Sterilized equipment was taken to two sites at the Southeast Kansas Biological Station to collect soil samples from Monahan Outdoor Education Center (MOEC) and O'Malley properties, a part of southeast Kansas Biological Station. These two geographically unique locations were chosen based on different type of soil. The sampling was completed in October of 2015.

A total of 20 soil samples were collected using sterile equipment: a soil corer scoop, 50 ml Falcon tubes, sterile latex gloves, and a temperature probe. Two sites were sampled for a total of 10 samples from each site on fall season.



A **B.**

Fig.1 Monahan (A) and O'Malley (B) properties

Quantitative analysis of soil samples

The C: N ratios were based on 10 samples from each site. Soils were sieved and air dried. The analysis was performed at the Kansas State University Soil Laboratory on a LECO CN 2000 combustion analyzer using standard methods. (Recommended Chemical Soil Test Procedures, 1998). The soils at our reclaimed and undisturbed sites were not different, with C: N ratios around 12:1 at both sites. Therefore, any differences that might be found in the microbial communities at our sites cannot be explained by C: N ratios.

Soil DNA extraction

DNA was extracted from the soil sample using Power Soil DNA isolation Kit. This involved the following distinct steps:

0.25g of soil samples were added to the Powering of Bead Tubes provided, the content was gently vortex. The solution was checked for any form of precipitation, and then heated at 60 degrees till all the precipitates were dissolved. 60 microliter of the solution c1 was then added and vortexed gently. Power Bead Tubes were then secured horizontally using the MO BIO Vortex Adapter tube holder for about 10 minutes. The resultant supernatant was then transferred to a clean 2ml Collection tube. 250 microliter of solution C2 was added and then vortexes for 5seconds. The tube was centrifuged at room temperature for about 1minute at 10,000 times gravity. Pellets were avoided and transfer of 60 microliters of supernatant to the Collection Tubule was done. After addition of C6 was done, the tube was centrifuged at room temperature for about 30 seconds The DNA tube became ready for use subsequently.

Polymerase chain reaction (PCR) Procedure

PCR reaction mixture of 20 µl included: one µl of bacterial DNA as template, 20 pmole of each primer (0.5 µl) 27F, (0.5 µl) 1492R (Lane et al, 1991, Biosynthesis Co.)

Table 1. Details of PCR primers used in this study to amplify selected genes from soil DNA

Target for amplification	Primer sequence	Amplicon Length	Reference
Ar4F[a] (8–25)[b] Ar958R[a] (958–967)	F TCY GGT TGA TCC TGC CRG R YCC GGC GTT GAV TCC AAT T	1000bp	13
Fungal specific ITS	F 5' GCGGAAGGATCATTACTGAG R 5' GGGTATCCCTACCTGATCCG	500	14
27F 1492R	AGAGTTTGATCCTGGCTCAG GGTTACCTTGTTACGACTT	1500bp	14

10 µl of Promega PCR Master Mix (Madison, WI), and 8 µl sterile water. The PCR reaction mixture was denatured at 95^0 C for a period of 3 minutes before 29 cycles of denaturing at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for one minute. The last extension step was at 72°C for 10 minutes. The infinite hold was set at 4°C. The composition of Promega PCR mastermix is (Taq DNA Polymerase, dNTPs, MgCl2 and reaction Buffers)

Agarose Gel Electrophoresis

Gel media was prepared from 1% agarose and 1X Tris base, acetic acid and EDTA buffer (TAE) and 3.5 µl (recommended: 1 µl per 20 ml gel) iNtRON RedSafe Nucleic Acid Staining Solution (20,000x). The gel was poured into a Fisher Biotech Electrophoresis Mini Horizontal Unit then left to solidify before placed it in 1X TAE buffer with ¼ inch to ½ inch TAE buffer covering the agarose gel.

Loading samples: A Bullseye 100 base pair (bp) DNA ladder (MIDSCI) was pipetted in the first well in the amount of five µl. The samples were run at 80 volts for 75 minutes. The gel was removed from the buffer solution to visualize under ultraviolet light Electrophoresis Systems 312 Transilluminator.

PCR product purification by DNA Clean & Concentrator kit (Zymo Research) was used to purify PCR amplicons and quantitation using Nanodrop.

PCR Cloning

Vector featured T-Overhangs for easy PCR cloning: The PGEM-T and PGEM-T Easy vectors were linearized with a single 3 terminal thymidine at both ends. The T-overhangs at the insertion site greatly improved the efficiency of litigation of PCR products by preventing recircularization of vector and provided a compatible overhang for PCR generated by certain thermostable polymerases.

Blue/white selection of recombinants: the PGEM-T and PGEM-T Easy vectors were high–copy–number vectors which contained the T7 and SP 6 RNA polymerase promoters flanking multiple cloning regions within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allowed identification of recombinants by blue/white screening on indicator plates.

Choice of a restriction site for release insert: Both the PGEM-T and PGEM-T Easy vectors contained numerous restriction sites within the multiple cloning regions. The PGEM-T Easy vector multiple cloning regions was flanked by recognition sites for the restriction enzymes EcoRI, BstZI, and NotI, which provided three single-enzyme digestions for the release of the inserts. The PGEM-T-vector cloning region was flanked by recognition sites for the enzyme BstZI. Alternatively, double-digestion might be used to release the insert from either vector.

Rapid Ligation: the PGEM-T and PGEM-T Easy vector systems were supplied with 2X Rapid Ligation Buffer. Ligations reactions using this Buffer might be incubated for 1hour at room temperature. The incubation period might be extended to increase the number of colonies after transformation. An overnight incubation at 4°C produced the maximum number of transformants.

Ligation Protocol

PGEM-T or PGEM-T Easy vector and Control Insert DNA tubes were briefly centrifuged to collect the content at the bottom of the tube. Ligation reaction was then set up using 0.5ml tubes that are known to have the low DNA- binding capacity for instant VWR Cat. 20170-310. The 2x rapid ligation buffer was then vortexed vigorously before use. The reactions were mixed by pipetting then incubated for 1 hour at the room temperature. To attain maximum number of transformants, the reaction can be incubated at 4 degrees overnight.

Optimizing Insert: Vector Molar Ratios

The PGEM-T and PGEM-T Easy vector systems were optimized using 1: the 1molar ratio of the Control Insert DNA to the vectors. Ratio optimization might be necessary if the initial experiments with PCR product were suboptimal. The

concentration of PCR product was estimated by comparison to DNA mass standards on a gel or by using fluorescent assay.

Screening Transformants for inserts

Successful cloning of an insert into PGEM-T or PGEM-T Easy vector interrupt the coding sequence of β-galactosidase; recombinant clones could be identified by color screening on indicator plates. Characteristics of the PCR cloned into vectors can affect the ratio of blue: white colonies obtained. Clones containing PCR products produced white colonies, but the blue colonies resulted from PCR fragments that are cloned in-frame with the LacZ genes. Results obtained with the Control Insert DNA might not be representative of those achieved with PCR product.

Isolation of bacterial isolates from fresh soil samples. 1g soil re suspended in 10 ml 0.9% saline. Soil were then diluted from (10^{-1} to 10^{-8}). 100 µl of various dilutions. spread plated on Tryptic Soy Agar (TSA). The plate were Incubated at 28°C for 24-72 hours. Morphologically different colonies were selected and re-streaked on TSA for further analysis.

Restriction Fragment Length Polymorphism (RFLP)

The following experiment employed two major techniques of amplifying and separation of different but specific DNA fragments. The polymerase chain reaction technique is on the major technique used in replication of a specific DNA codon for better examination. The required strand is primed using fluorescent primer for easy recognition. The Restriction Fragment Length Polymorphism (RFLP) is a technique used in the differentiation of the different DNA strands by the point each strand is cleaved by the restricting enzyme, (Fong, Ho, & Poon, 2017).

The 16S rRNA gene sequence has a specific portion or fragment known as conserved region which can easily be replicated numerously using the polymerase chain reaction method. The specific endo-nucleases enzyme used were the *BsuRI* and *Hhal*, which are restriction enzymes. The restriction digestion reaction was 20 µl contains green buffer, restriction enzyme, water and PCR DNA. The reaction was digested at 37℃ for one hour. The cleaved fragments were separated using the method of electrophoresis on 2.5% agarose gel.

Table2. Information on restriction enzymes used in this study.

Restriction enzymes used for RFLP analysis	**Recognition sequences (down arrow indicates cut site) Sequence**	**Source**
BsuRI	5' G G↓C C 3' 3' C C↑G G 5'	Thermo Scientific
HhaI	5' G C G↓C 3' 3' C↑G C G 5'	Thermo Scientific

Antibiotic susceptibility testing by Kirby-Bauer disc diffusion assay

The Mueller-Hinton mixture included two grams of beef extract, 17.5 grams of casamino acids, 1.5 grams of starch and 15 grams of agar, suspended in 1000 milliliters of distilled water. A sterile cotton swab was dipped into the suspension and rolled onto Mueller-Hinton agar, completely covering the plate. Once inoculated, each plate sat for five minutes to dry prior to adding the BBL Sensi-Disc Antimicrobial Susceptibility test discs. Each sample was streaked onto two plates and divided evenly down the middle. On each side, an antibiotic disk was placed using sterilized forceps that had been dipped into alcohol and passed through the Bunsen burner prior to placing each

antibiotic disc. The following disks were used for all samples (Ampicillin 10µg/disk, Ciprofloxacin 5µg/disk, Erythromycin 15µg/disk, Tetracycline 30µg/disk, Polymyxin B 300U/disk). Once the disks were placed, the plates were allowed to dry for five minutes prior to inversion and placement in the incubator at 37°C for 48 hours. Once 48 hours had passed, the zones of inhibition surrounding each disc were measured in millimeters and recorded.

Chapter III

Results and Discussion

Most of the ecosystems are declining due to experience of erosion, low productivity and poor water quality caused by clearing of forest intensive agricultural production and the use of land resources for reasons that are unsustainable (Kennedy and Smith, 1995). The study of the populations of the microbes and their responses to stress can enable one to understand the functionality of the ecosystems as it is governed by soil microbial dynamics.

Previously, the identification and characterization of microbes that contaminate water and soil was limited to microbes that can be cultured. However the use of molecular techniques nowadays has made it easy to study the microbes even without culturing and it has led to invention of new microbes. The extraction of nucleic acids from contaminated sites by bacteria and the subsequent amplification by polymerase chain reaction (PCR) have greatly helped in assessing the changes in microbial structure (Malik, Seidu, et al, 2008).

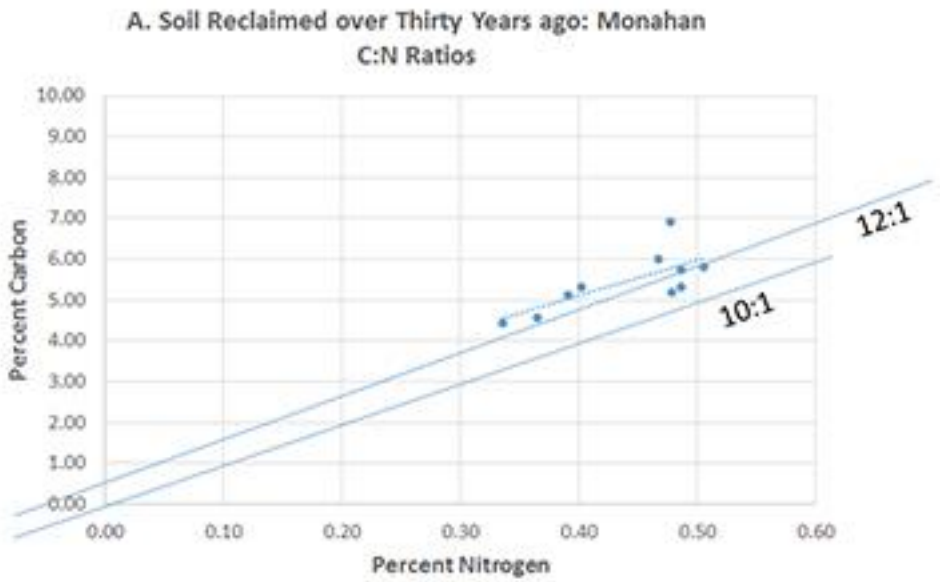


Fig. 2A. Carbon and nitrogen quantitative estimation of soil samples

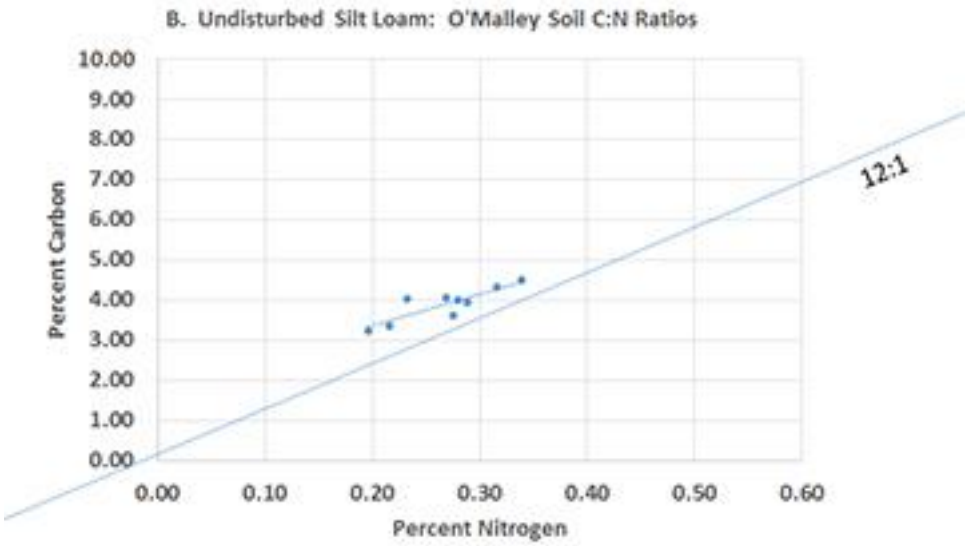


Fig. 2B. Carbon and nitrogen quantitative estimation of soil samples

Most of soil organic matter consist of carbon and nitrogen among others. Fine-silt-size particles that was separated from inside macro aggregates contained the highest percentage of total soil C and N (Cambardella, and Elliott, 1994,).

Our hypothesis predicted that the newer soil at the reclaimed (Monahan) site would have a distinctly different microbial community than the original, undisturbed soil. If our data supported that hypothesis, differences in C: N ratios could suggest a

reason for different communities. The C: N ratios for bacterial cells rarely exceeds 7:1, with fungal cells requiring values as high as 15:1 (Neidhardt 1996). However, soil testing for carbon and nitrogen at the Kansas State Soil Testing Laboratory did not support significantly different C: N ratios.

Table 3. DNA concentration for various soil samples extracted using power soil DNA isolation kit

Sample#	O1	O2	O3	O4	M1	M2	M3	M4
ng/uL	10.3 9.6	44.5	32.1	32.8	11.8 14.3	30.4	25.4	22.8
260\280	1.79	1.82	1.86	1.83	1.86	1.85	1.79	1.84

O1-4= O'Malley

M1-4= Monahan

Quantity and purity of extracted DNA was appropriate for carrying out further molecular processes. The expected purity is 1.8.

Table 4. DNA concentration for purified PCR products.

The larger DNA fragments can be used as a better DNA template for further PCR amplification of the desired genes.

Samples	ng/µl	260/280
O1	63.1	1.33
O2	51.6	1.32
O3	80.7	1.38
O4	46.6	1.27

O5	106.3	1.31
O6	26.7	1.05
O7	17.1	0.79
O8	35.8	0.93
O9	17.0	0.74
O10	38.0	1.02
O11	66.7	1.34
O12	88.7	1.46
O13	79.3	1.39
O14	28.6	1.02
O15	72.1	1.37
M1	35.1	1.47
M2	34.6	1.10
M3	22.2	0.87
M4	23.5	0.71
M5	31.2	1.03
M6	54.9	1.41
M7	10.1	0.72
M8	17.2	0.75
M9	15.2	0.70
M10	15.1	0.75
M11	23.1	0.93
M12	40.4	1.16
M13	16.2	0.84

M14	27.9	1.14
M15	17.4	0.93

- O'Malley and Monahan soil yield larger DNA fragments sheared. The quantity of DNA was also determined by nanodrop lite.

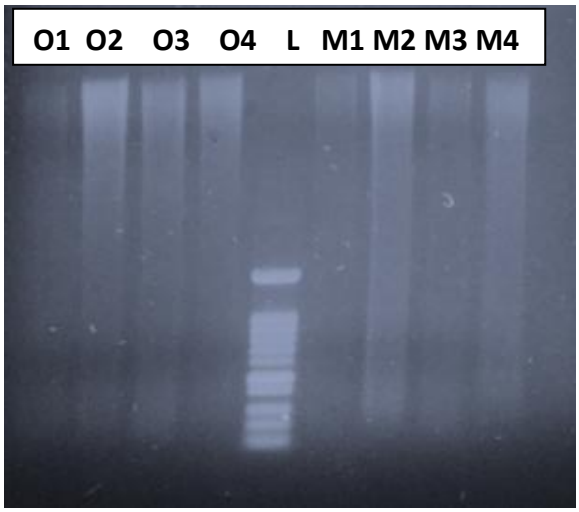


Fig. 3. Agarose gel showing isolated soil DNA from Monahan and O'Malley. O1-4 is O'Malley original, soil M1-4 is Monahan reclaimed soil and L is 100 bp DNA ladder. MO Bio Power Soil DNA Isolation protocol could yield intact DNA with minimum shear from both soil sources.

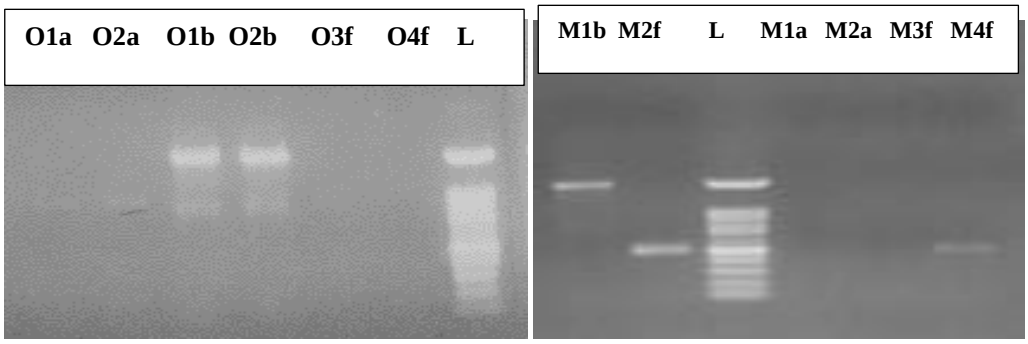


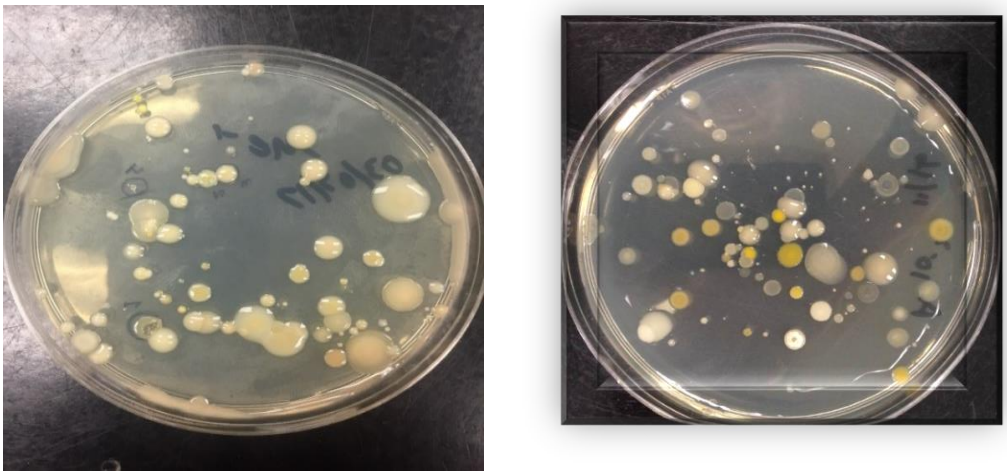
Fig.4. Amplicons for bacterial gene (b) (O1b, O2b, M1b) = 1500bp fungal gene (f) (M2f, M4f) = 500bp archaeal gene (a) (O1a, O2a) = 1000bp

Recently, the culture independent methods have been used to analysis microbial communities, although it has raised many questions whether uncultured samples are necessary in determination of the impact of anthropogenic stress on indigenous communities. To scrutinize this, soil samples were taken from a site

witchy metal contamination and the community was assessed in various approaches. There were small differences in microscopic epifluorescence bacterial counts but. Denaturing gradient gel electrophoresis (DGGE) profiles of 16S rRNA gene fragments (16S-DGGE) amplified directly from soil samples were highly similar. The culturable communities from each sample were compared by 16S-DGGE of plate washes and by fatty acid profiling of individual isolates and each of these approaches showed that there was considerable differences between the compositions of the culturable communities from each sample. DGGE bands from both culture-based and culture independent approaches were squeezed and compared and the results indicated that metal contamination did not have a significant effect on the total genetic diversity present but affected physiological status, so that the bacteria able to respond to lab culture and their distribution were altered.

The amplicons were purified and ligated to pGEMT vector; however, the cloning kit did yield numerous clones on selective plate. Since the cloning of the PCR products did not work out so far, the project also focused on culturable bacterial diversity as an alternative approach. Following dilution plating procedure bacterial strains were isolated from O'Malley soil samples on Tryptic Soy Agar (TSA) Mewntion the concentration of bacteria in both sample sources

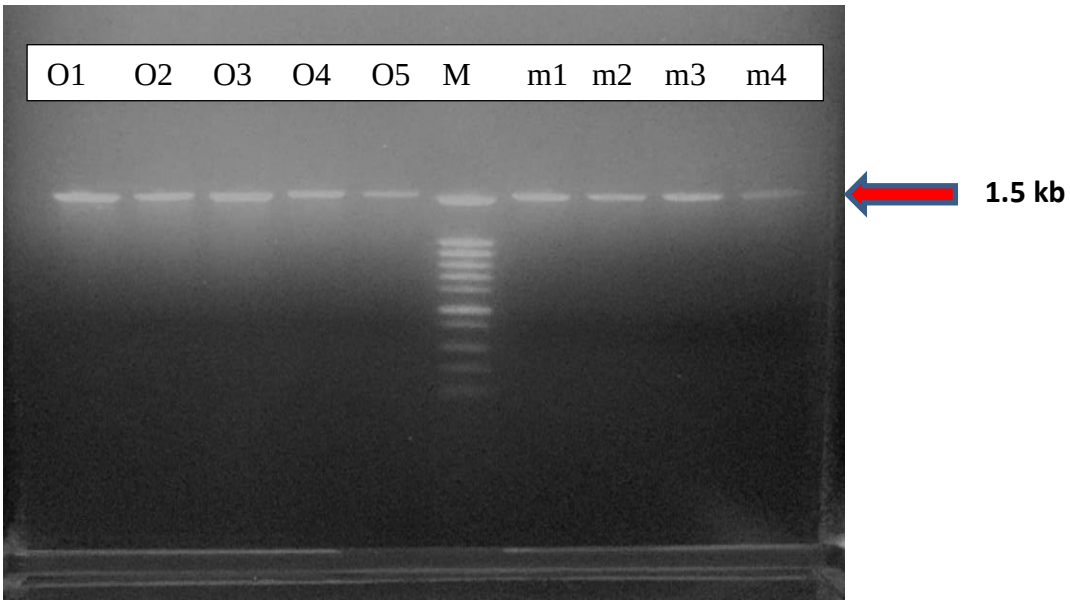
On individual isolates PCR was performed to amplify 16S rRNA gene. Further the amplicons were digested using name the enzymes restriction enzymes. The RFLP was also performed on isolates from Monahan soil that was obtained in a parallel study in the lab.The RFLP patterns were compared



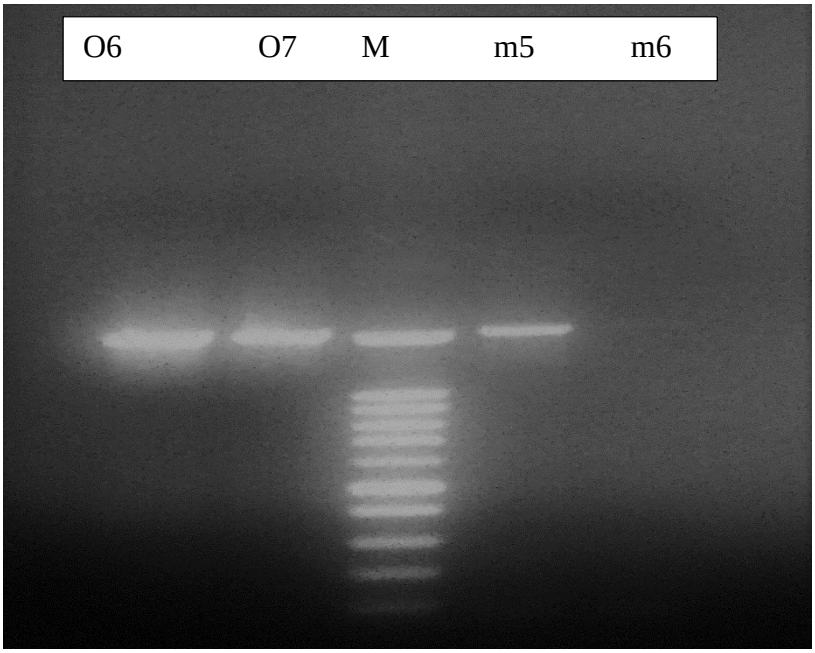
A **B**

Fig.5. Bacterial isolates from fresh soil samples. the colonies were white, creamy, round and mucus. Concentration of bacteria in O'Malley samples A: 102-104 CFU/ gram of soil Monahan samples B: 102- 108 CFU/ gram of soil.

Up to 30 morphologically (colony color, shape) different bacterial isolates were selected from the TSA media, re-streaked and preserved for further analysis.

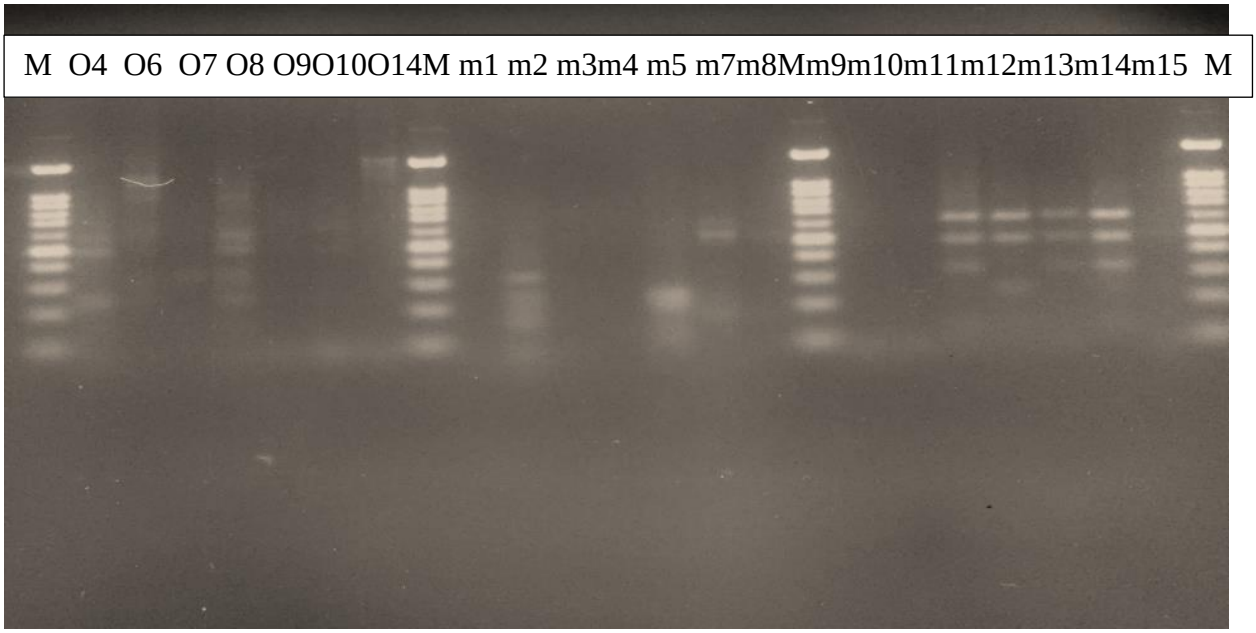


A

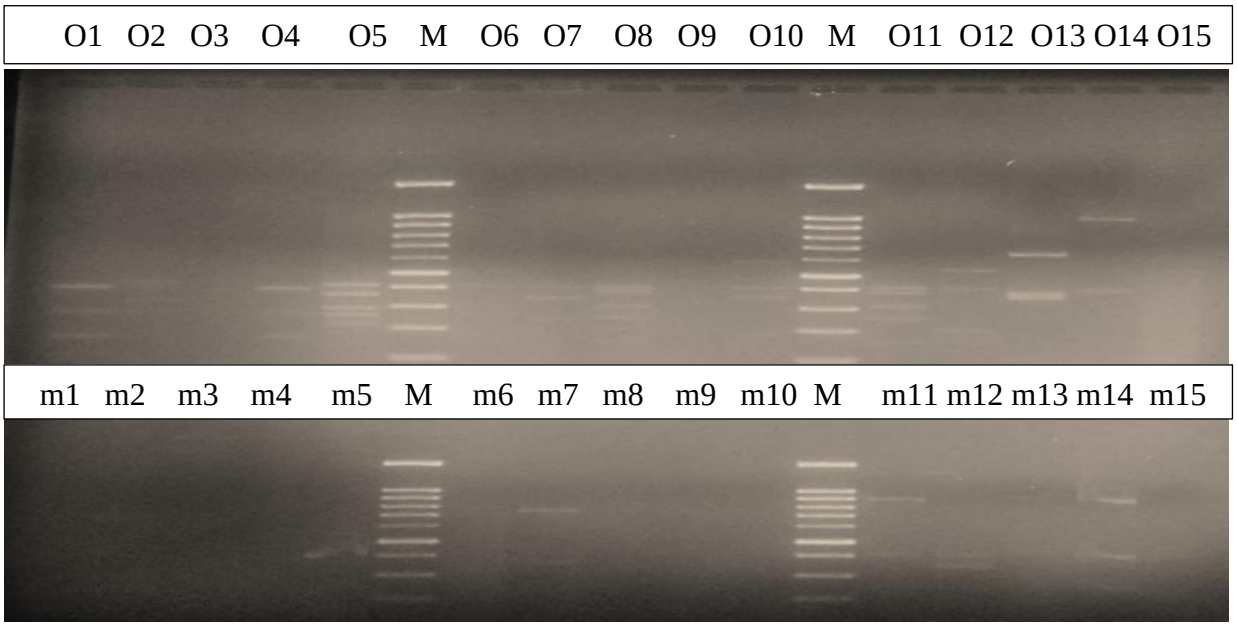


B

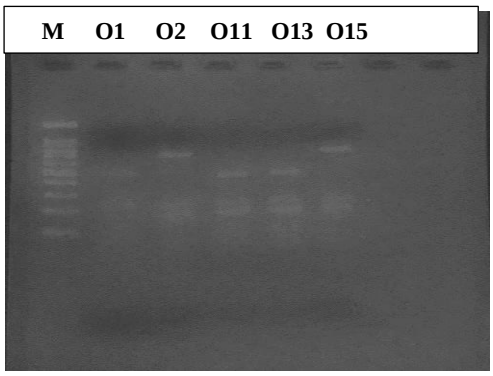
Fig.6. 16S rRNA gene amplicon for RFLP analysis 1.5 kb amplicon was PCR amplified from all the isolates to be tested and they were purified from using the DNA purification kit for setting up RFLP digestion

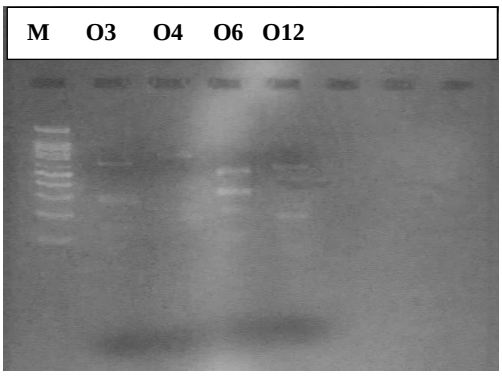


A

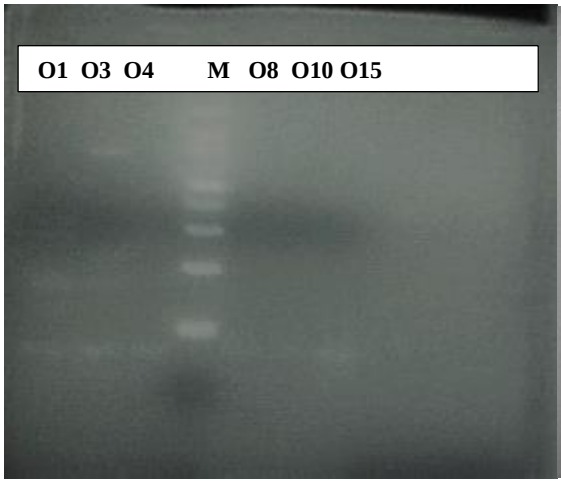


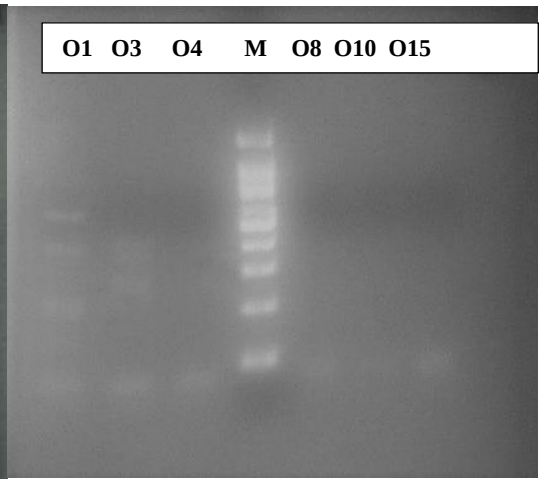
B





C D





E F

Fig. 7 RFLP analysis on several isolates from O'Malley and Monahan on 2.5% agarose gel

Figures A, B, C, D, E and F show comparison of RFLP patterns of isolates from two different soil sources. Evidently, the isolates from Monahan and O'Malley showed differences in band pattern indicating variation in the 16S rRNA sequences. Gel-band reading software will be used to generate phylogenetic trees.

Table 5 illustrates draft of various band lengths.

Table 5a. RFLP pattern comparison based on band length

BsuRI	O'Malley														
Band Length	**O1**	**O2**	**O3**	**O4**	**O5**	**O6**	**O7**	**O8**	**O9**	**O10**	**O11**	**O12**	**O13**	**O14**	**O15**
1500															
														1250	
1000								1000							
					950										
900															
800		800		800							800		800		
			850												
700				700			700					700			
600															600
500															500
					450										
400															
							450					450			
300			300					300					300		
											350				350
200		200					200				200	200	200	200	
		250													
100					100			100							
Total	**ND**	**1250**	**1150**	**1500**	**1500**	**ND**	**1350**	**1400**	**ND**	**ND**	**1350**	**1350**	**1300**	**1450**	**1450**

Table 5b. RFLP pattern comparison based on band length

BsuRI Monahan

Band Length	m1	m2	m3	m4	m5	m6	m7	m8	m9	m10	m11	m12	m13	m14	m15
1500															
1000															
900															
800							800								
700						700	700			700					
					750										
600								600			600	600	600		600
		650													
500			500								500	500	500	500	
400	400			400	400					400				400	
															450
300	300	300	300	300				300		300	300		300	300	
		350			350	350						350			
200	200	200	200	200		200		200							200
	250		250	250				250						250	
100	100		100	100				100		100					100
Total	**1250**	**1500**	**1350**	**1250**	**1500**	**1250**	**1500**	**1450**	**ND**	**1500**	**1400**	**1450**	**1400**	**1450**	**1350**

Table 5c. RFLP pattern comparison based on band length

HhaI O'Malley

Band Length	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10	O11	O12	O13	O14	O15
1500															
1000														1000	
900															
800	800														
700						700	700								
				750											
600								600		600		600			
500												500	500	500	
400	400			400	400	400		400		400	500				
					450		450			450	450		450		
300	300				300			300			300		300		
					350	350						350			
200				200			200								
100															
Total	**1500**	**ND**	**ND**	**1350**	**1500**	**1450**	**1350**	**1300**	**ND**	**1450**	**1250**	**1450**	**1250**	**1500**	**ND**

Table 5d. RFLP pattern comparison based on band length

Hhal Monahan

Band Length	m1	m2	m3	m4	m5	m6	m7	m8	m9	m10	m11	m12	m13	m14	m15
1500															
1000															
900	900										900			900	
800					800				800						800
700			700	700						700					
600												600			
500			500												
400	400				400							400		400	
										450					
300				300							300	450			300
									350						
200			200							200					200
				250	250				200						
100				100					100	100					100
Total	**1300**	**ND**	**1400**	**1350**	**1450**	**ND**	**ND**	**ND**	**1450**	**1450**	**1200**	**1450**	**ND**	**1300**	**1400**

Table 6. Summary of RFLP pattern comparison based on band length

O'Malley	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10	O11	O12	O13	O14	O15
HhaI	1500	ND	ND	1350	1500	1450	1350	1300	ND	1450	1250	1450	1250	1500	ND
BsuRI	ND	1250	1150	1500	1550	ND	1350	1400	ND	ND	1350	1350	1300	1500	1450

Monahan	m1	m2	m3	m4	m5	m6	m7	m8	m9	m10	m11	m12	m13	m14	m15
HhaI	1300	ND	1400	1350	1450	ND	ND	ND	1450	1450	1200	1450	ND	1300	1400
BsuRI	1250	1500	1350	1250	1500	1250	1500	1450	ND	1500	1400	1450	1400	1450	1350

16S rRNA gene clone was used to study characterize bacteria communities under phylogenetic- and species-based frameworks. Labelled DNA was used to assess the composition of the active taxa in the active sites and it was found that most of the sites were dominated by α- *Proteobacteria*, followed by acidobacterial and betaproteobacterial sequences (Dimitriu, Pedro, and Susan, 2010).

Perennial ryegrass and alfalfa have the ability to increase the number of rhizosphere bacteria in the hydrocarbon-contaminated soils as well as the bacteria with the ability to degrade petroleum, (Kirk, Jennifer L., et al, 2005). Eco-Biology plates cannot be able to detect the variation in metabolic diversity between rhizosphere samples, however, the analysis of PCR-amplified partial 16S rDNA by use of denaturing gradient gel electrophoresis (DGGE) sequences indicated a shift in the bacterial community in the rhizosphere samples.

Heavy metals in bio solids may permanently degrade the microbial decomposers of agricultural soils. Ribosomal DNA restriction analysis can be used to compare the diversity of zinc-contaminated soil with that of a control soil from a sewage sludge (Moffett, Bruce F., et al, 2003). The toxicity of zinc lowers the diversity of bacteria.

An experiment was done on the long term exposure of mercury in different sites along a pollution gradient. The amount and bioavailable mercury was negatively correlated t the distance from the point of contamination. The bacteria and protozoan population was noted to decrease in the contaminated areas whereas there was no difference in fungus biomass. The bacteria species that remained in large proportions had an ability of resistant and fast growing (Müller, Anne Kirstine, et al, 2001). Denaturing gradient gel electrophoresis (DGGE) on the amplified 16S rDNA sequences that was got from these bacteria also showed that there was decreased diversity along the mercury gradient.

Table 7. antibiotic susceptibility profile of O'Malley (O1-O15) and Monahan (M1-M15) isolates.

ID.	TET	AMP	PB	CIP	ERY
O1	28±1	31±1	20±1	27±1	29±1
O2	26±1.5	22±1	27±1.5	25±1	30±0.5
O3	26±1	29±2	33±1	30±1	0
O4	32±1	34±1	31±1	33±1	0
O5	27±2	24±1	22±1.5	23±1	26±1
O6	33±1	21±1.5	23±1	24±1	28±1.5
O7	34±1.5	24±0.5	34±1	35 ±1.5	33±1
O8	29±1	30±1	22±1	43±1	0
O9	31±1	35±0.5	28±1	35±1	30±2
O10	27±2	32±1	29±1	31±0.5	25±1
O11	32±2	27±1	34±2	28±1	30±2
O12	43±1	38±1	25±1	32±1	29±1
O13	33±0.5	39±1	26±0.5	32±1	31±0.5
O14	23±0.5	0	28±1	24±0.5	23±1
O15	30±1	33±1	22±1	21±1	33±1.5
M1	33±1	0	29±1	32±1	23±1
M2	25±2	26±1	22±1	22±1	32±1
M3	24±1	0	21±0.5	33±1	27±1
M4	40±1	21±1	25±1	23±1.5	28±1.5
M5	30±1	30±1	38±1	22±1	31±1
M6	22±1.5	22±1	22±2	31±1	33±1
M7	21±1	31 ±1	30±1	25±0.5	27±2
M8	29±1	35±1	28±1	22±1	26±1
M9	30±1.5	22±1	35±1	31±1	22±1
M10	32±1	30±1	29±1	34±2	25±0.5
M11	21±1	26±1	30±1	28±1	21±1
M12	25±1	22±1	22±2	21±1	33±1
M13	30±2	33±1	35±1	34±1.5	26±0.5
M14	21±1	0	28±1	30±1	0
M15	26±1	0	22±1	22±1	0

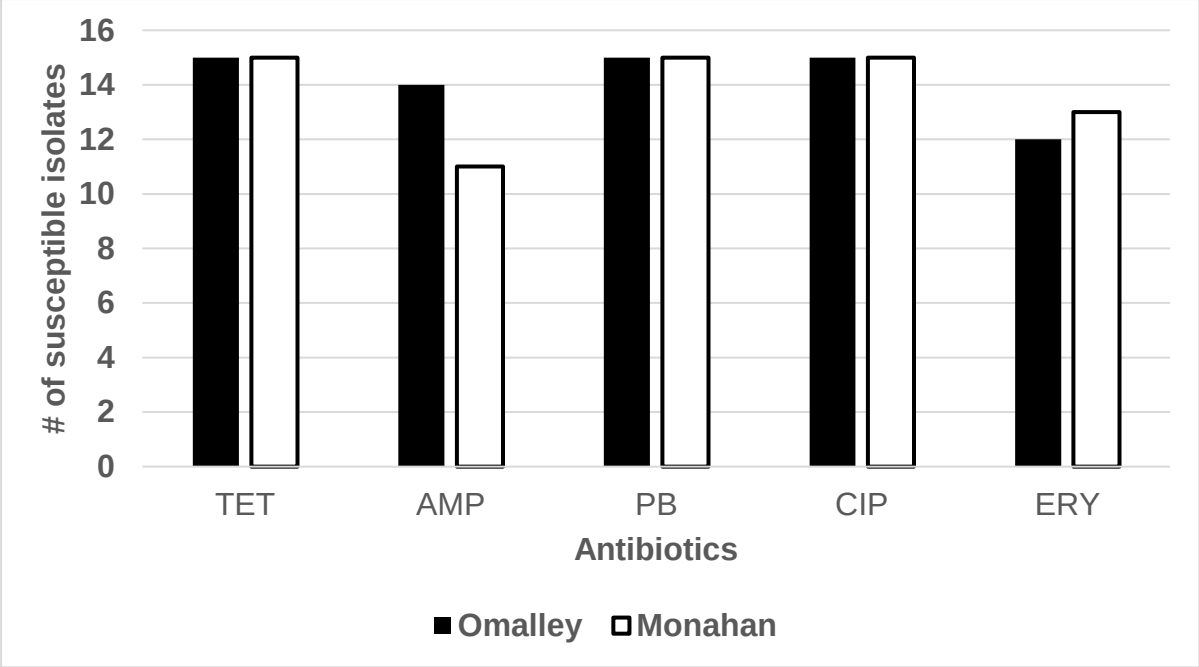


Fig.9 Antibiotic susceptibility profile

There was no significant difference in the antibiotic susceptibility profile of these isolates. This result indicated that the microbes involved in the biogeochemical processes not necessarily show greater resistance to antibiotics. Pristine O'Malley soil isolates or Monahan isolates showed resistance to fewer antibiotics that are assumed to be intrinsic resistance showed by the bacterial isolates.

One of the ways to ensure the differentiation of soil microbes is to use RFLP a method that would conserve the sequence of I6S r RNA genes. 1.5 kb amplicon for 30 PCR amplified samples were tested by RFLP digestion. The samples were digested on 37℃ for one hour. The digestion enzyme cut the DNA fragment to a small pieces the need a high concentration of agarose to be visible under the ultraviolet. 2.5% of agarose gel have been used. 29 samples were positive and had a bright band. One thing that was evident is that digestion of the DNA fragment by the restriction of enzymes (BsuRI, HhaI) had a different outcome in pattern Length.

Chapter IV

Conclusions

Data showed that bacterial, archaeal and fungal communities were present in both types of soil at the molecular level. Cloning of specific genes failed, so the approach was shifted from non-culturable DNA based method to culturable isolates. Restriction-fragment length polymorphism (RFLP) reflected a qualitative comparison of the microbial communities. Concentration of bacteria in O'Malley soil samples were within the range 10^2-10^4 CFU/gram of soil while for Monahan 10^2-10_8 CFU/gram. Altogether, 30 morphologically different bacterial colonies from Monahan and O'Malley were selected for RFLP analysis; there were no remarkable difference in community structure based on RFLP band pattern analysis. In addition, antibiotic susceptibility profile of these isolates were not different between two sources. C: N ratio of these two types of soil samples were comparable, and are unlikely to be a source of variation between these microbial communities.

Although we hypothesized that there would be differences in the microbial communities of the two soils, we did not find differences in the *bacterial* community. Therefore, our results did not support our original hypothesis. However, further analysis of the fungal and archaeal communities will need to be completed before we can assume that the soils truly are the same in this respect. It is also possible that differences in the microbial community would be more pronounced in other seasons of the year, or that the communities in reclamations are different at first, becoming

more and more similar as time passes. Because this initial study of a 30-year-old soil did not reveal the anticipated differences, we suggest that similar analyses should be focused on the earlier stages of a reclamation at a future site. Studies like this one are needed to determine the most effective timing of treatments (e.g. microbial innoculants or additives) to enhance soil development. If site managers can learn how long it takes a reclaimed soil to assume the characteristics of native soil, the can be better prepared to learn how and when managing the soil microbial community can improve or accelerate soil development.

References

1-Ahmad, I., Ahmad, F., & Pichtel, J. (Eds.). (2011). *Microbes and microbial technology: Agricultural and environmental applications*. Springer Science & Business Media.

2-Bluth, M. J., & Bluth, M. H. (2013). Molecular pathology techniques. *Clinics in laboratory medicine*, *33*(4), 753-772.

3-Dickie, I. A., Xu, B., & Koide, R. T. (2002). Vertical niche differentiation of ectomycorrhizal hyphae in soil as shown by T-RFLP analysis. *New phytologist*, *156*(3), 527-535.

4-Dineen, S. M., Aranda, R., Anders, D. L., & Robertson, J. M. (2010). An evaluation of commercial DNA extraction kits for the isolation of bacterial spore DNA from soil. *Journal of applied microbiology*, *109*(6), 1886-1896.

5-Dong, W. Y., Zhang, X. Y., Dai, X. Q., Fu, X. L., Yang, F. T., Liu, X. Y., & Schaeffer, S. (2014). Changes in soil microbial community composition in response to fertilization of paddy soils in subtropical China. *Applied soil ecology*, *84*, 140-147.

6-Mallett, X., Blythe, T., & Berry, R. (Eds.). (2014). *Advances in forensic human identification*. CRC Press.

7-Narendrula-Kotha, R., & Nkongolo, K. K. (2017). Microbial Response to Soil Liming of Damaged Ecosystems Revealed by Pyrosequencing and Phospholipid Fatty Acid Analyses. *PloS one*, *12*(1), e0168497.

8-Petric, I., Philippot, L., Abbate, C., Bispo, A., Chesnot, T., Hallin, S., & Lindström, K. (2011). Inter-laboratory evaluation of the ISO standard 11063 "Soil quality—Method to directly extract DNA from soil samples". *Journal of microbiological methods*, *84*(3), 454-460.

9-Philippot, L., Hallet, S., Chaussod, R., Germon, J. C., Soulas, G., & Catroux, G. (2001). DNA extraction from soils: old bias for new microbial diversity analysis methods. *Applied and environmental microbiology*, *67*(5), 2354-2359.

10-Rahmani, S., Forozandeh, M., Mosavi, M., & Rezaee, A. (2006). Detection of bacteria by amplifying the 16s rRNA gene with universal primers and RFLP. *Medical journal of the Islamic Republic of Iran (MJIRI)*, *19*(4), 333-338.

11-Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., & Sahl, J.W. (2009). Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and environmental microbiology*,*75*(23), 7537-7541.

12-Wakelin, S. A., Macdonald, L. M., Rogers, S. L., Gregg, A. L., Bolger, T. P., & Baldock, J. A. (2008). Habitat selective factors influencing the structural composition and functional capacity of microbial communities in agricultural soils. *Soil biology and biochemistry*, *40*(3), 803-813.

13- Jurgens, G. (2000). Identification of novel Archaea in bacterioplankton of a boreal forest lake by phylogenetic analysis and fluorescent in situ hybridization. *FEMS microbiology ecology*, *34*(1), 45-56. doi:10.1016/s0168-6496(00)00073-8

14-Yeates, C., Gillings, M. R., Davison, A. D., Altavilla, N., & Veal, D. A. (1998). Methods for microbial DNA extraction from soil for PCR amplification. *Biological procedures*, *1*(1), 40.

15- Scheidegger, Emd, Sap Fracalanzza, Lm Teixeira, and P. Cardarelli-Leite. (2009). RFLP analysis of a PCR-amplified fragment of the 16S rRNA gene as a tool to identify Enterococcus strains. *Memórias do Instituto Oswaldo Cruz*, *104*(7).

16- Zhao, L., Zhihong M., Yunxia L., Anxiang L., Jihua W., and Ligang P. (2011). Molecular methods of studying microbial diversity in soil environments. *Computer*

and computing technologies in agriculture IV IFIP Advances in information and communication technology.

17- Guimarães, D. V., Gonzaga, M. I. S., da Silva, T. O., da Silva, T. L., da Silva Dias, N., & Matias, M. I. S. (2013). Soil organic matter pools and carbon fractions in soil under different land uses. *Soil and tillage research*, *126*, 177-182.

Batjes, N. H. (2014). Total carbon and nitrogen in the soils of the world. *European* j*ournal of soil science*, *65*(1), 10-21.

18-Mooshammer, M., Wanek, W., Hämmerle, I., Fuchslueger, L., Hofhansl, F., Knoltsch, A., & Keiblinger, K. M. (2014). Adjustment of microbial nitrogen use efficiency to carbon: nitrogen imbalances regulates soil nitrogen cycling. *Nature communications*, 5.

19-Neidhardt, F.C., et al., eds. (1996). Escherichia coli and Salmonella typhimurium - *Cellular and Molecular Biology*, 2nd ed. Am. Soc. Microbial, Washington, DC.

20-Cambardella, C. A., & Elliott, E. T. (1994). Carbon and nitrogen dynamics of soil organic matter fractions from cultivated grassland soils. *Soil science society of America journal*, *58*(1), 123-130.

21-Dimitriu, P. A., & Grayston, S. J. (2010). Relationship between soil properties and patterns of bacterial β-diversity across reclaimed and natural boreal forest soils. *Microbial ecology*, 59(3), 563-573.

22-Kennedy, A. C., & Smith, K. L. (1995). Soil microbial diversity and the sustainability of agricultural soils. In *The significance and regulation of soil biodiversity*. Springer: Netherlands,75-86.

23-Kirk, J. L., Klironomos, J. N., Lee, H., & Trevors, J. T. (2005). The effects of perennial ryegrass and alfalfa on microbial abundance and diversity in petroleum contaminated soil. *Environmental pollution*, *133*(3), 455-465.

24-Malik, S., Beer, M., Megharaj, M., & Naidu, R. (2008). The use of molecular techniques to characterize the microbial communities in contaminated soil and water. *Environment international*, *34*(2), 265-276.

25-Moffett, B. F., Nicholson, F. A., Uwakwe, N. C., Chambers, B. J., Harris, J. A., & Hill, T. C. (2003). Zinc contamination decreases the bacterial diversity of agricultural soil. *FEMS microbiology ecology*, *43*(1), 13-19.

26-Müller, A. K., Westergaard, K., Christensen, S., & Sørensen, S. J. (2001). The effect of long-term mercury pollution on the soil microbial community. *FEMS microbiology ecology*, *36*(1), 11-19.